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MONOCLONAL ANTIBODIES TO GLUTATHIONE S-TRANSFERASE π—IMMUNOHISTOCHEMICAL ANALYSIS OF HUMAN TISSUES AND CANCERS

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Mouse monoclonal antibodies (MAb) have been generated against the anionic isozyme of human glutathione S-transferase (GST π). MAb AGST I can inhibit 50–70% of GST π enzymatic activity and reacts with a 3-dimensional epitope which includes a putative glutathione binding site on GST π. A sandwich enzyme-immunoassay established using MAb AGST I and a polyclonal antibody displayed a sensitivity of 0.5 ng/ml. Immunohistochemical analysis of human tissues demonstrated marked increases in GST π levels in cancers of the brain, cervix, endometrium, colon, rectum and testis and in fibro- and chondrosarcomas.

Glutathione S-transferases (GST) are a family of enzymes which play an important role in cellular detoxification by catalyzing the conjugation of reduced glutathione to hydrophobic, electrophilic compounds (Mannervik, 1985). In addition, GST isozymes have been shown to bind non-catalytically to a number of lipophilic compounds and detoxify organic peroxides to less reactive alcohols (Jakoby, 1978). GST exist as homo- or heterodimers with subunit sizes of approximately M_r = 25,000 (Mannervik, 1985; Jakoby *et al.*, 1984). The members of this enzyme family exhibit broad and overlapping substrate specificities towards a large number of mutagenic, carcinogenic, and pharmacologically active substances (Boyland and Chasseaud, 1969; Chasseaud, 1979). The cytosolic forms of these enzymes are the products of 3 separate gene families (α, μ and π) and are distinguished on the basis of distinctive biochemical properties (Mannervik *et al.*, 1985).

The π class of GST in rodents, GST P, is reported to be one of the best markers for pre-neoplastic cells in some rat and hamster model systems of chemically induced hepatic and pancreatic cancers (Sato *et al.*, 1984; Satoh *et al.*, 1985; Tatematsu *et al.*, 1985; Moore *et al.*, 1985; Obara *et al.*, 1986). GST π enzymatic activity has been found in a variety of human cancers (Shea *et al.*, 1988), and GST π has been suggested to be a potentially useful immunohistological marker in pre-neoplastic lesions of the colon (Kodate *et al.*, 1986) and uterine cervix (Shiratori *et al.*, 1987). GST π has been implicated in the resistance of human tumors to antineoplastic drugs from the findings of increased levels of GST π in certain drug-resistant cell lines and tumors (Townsend and Cowan, 1989; Batist *et al.*, 1986; Deffie *et al.*, 1988; Kramer *et al.*, 1988; Moscow *et al.*, 1989).

As a first step in exploring the association between GST π, transformation, and drug resistance, we have produced MAbs to GST π. MAb to GST π have been used in structural analysis of this molecule as well as in the development of a sensitive enzyme immunoassay. We have also used the reagents to investigate immunohistochemically the distribution of this enzyme in a wide panel of normal human tissues and cancers.

MATERIAL AND METHODS

Polyclonal antisera to GST π and GST π peptides

GST π was obtained from placenta from a commercial source (Sigma, St. Louis, MO). GST π peptides corresponding to the following amino acid residues from the N-terminus of GST π were synthesized (Multiple Peptide Systems, San Diego, CA); peptide 1, 53–72; peptide 2, 66–85; peptide 3, 80–99; and peptide 4, 170–191. The GST π peptides were coupled to keyhole limpet hemocyanin (KLH) (CalBiochem, La Jolla, CA) using the cross-linker M-maleimido-benzyl-N-hydroxysuccinimide ester, MBS (Pierce, Rockford, IL). KLH was dissolved in 0.1 M phosphate buffer, pH 7.0, containing 2 mM MBS and incubated for 30 min at room temperature. Conjugated KLH was separated on a Sephadex G-25 column (Pharmacia, Piscataway, NJ) and incubated with an equal amount of peptide for 3 hr at room temperature. Male New Zealand white rabbits were initially immunized with 1 mg of GST π or peptides in Freund's complete adjuvant i.m. at 2 sites in each hind leg and with 200 μg of antigen in Freund's incomplete adjuvant for booster immunizations. When high serum antibody titers were detected by enzyme-linked immunosorbent assay (ELISA), animals were exsanguinated.

Production of MAb to GST π

GST π was either purified by affinity chromatography on S-hexylglutathione Sepharose (Sigma) from a doxorubicin-resistant human breast carcinoma cell line (Adr^RMCF-7,16) using a modification of the technique of Koskelo *et al.* (1981), or obtained from human placenta from a commercial source (Sigma). The purity of these preparations was monitored by enzymatic activity using 1 mM 1-chloro-2,4 dinitrobenzene and 1 mM GSH (Habig and Jakoby, 1981) and SDS-PAGE (Laemmli, 1970). For immunization, GST π (20–50 μg) was coupled to KLH and combined with Freund's complete adjuvant for the initial intraperitoneal (i.p.) immunization. Mice were immunized 3–5 times (i.p. with Freund's incomplete adjuvant) on bi-weekly schedules. Some immunization protocols used GST π and 1–2 mg of MAb 7C7 (IgM), a murine MAb to GST π. This immunization protocol was used in order to increase the probability of generating MAbs to epitopes on the molecule distinct from that recognized by MAb 7C7 (Barclay and Smith, 1986). When a strong anti-GST π serum titer was detected by ELISA, mice were boosted and 3 days later mouse splenocytes were isolated and fused with the mouse myeloma cell line, P3-X63-Ag8 (Giardina *et al.*, 1986).

Screening of hybridoma supernatants

Hybridomas were screened for reactivity with GST π by ELISA (Abrams *et al.*, 1983). The isotype subclasses of the hybridomas were determined by ELISA using mouse subclass-

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specific goat antibodies (Southern Biotechnology, Birmingham, AL).

Immunoblot analysis

Immunoblot analysis of GSH-affinity-purified GSTs was performed as described by Towbin *et al.* (1979). Samples were electrophoresed on 13.5% acrylamide gels (Laemmli, 1970). The gel was electro-blotted in a semi-dry apparatus (LKB, Gaithersburg, MD) for 1 hr at 100 mA constant current, using 48 mM Tris, 39 mM glycine, 5 mM EDTA transfer buffer. The blots were blocked in 5% non-fat milk for 1 hr and incubated overnight at 4°C with either 10 ml of MAb supernatant or purified rabbit antisera to GST π (10 μg/ml). Blots were washed with PBS and lanes exposed to MAb were then incubated with goat anti-mouse IgG (Organon Teknika-Cappel, Malvern, PA) for 2 hr followed by washing and addition of peroxidase-conjugated rabbit anti-goat IgG (Cappel). Lanes exposed to rabbit antisera were next incubated with peroxidase-conjugated goat anti-rabbit IgG (Cappel) for 2 hr. Washed blots were developed in 0.5 μg/ml 4-CL-1-naphthol/0.5 μl/ml 30% H_2O_2 in PBS/15% methanol.

Enzyme immunoassay for GST π

A sandwich method for enzyme immunoassay was employed using monoclonal and polyclonal antibodies to GST π. Briefly, purified MAb AGST 1 dissolved in PBS (100 ng/well) was added to 96-well plastic microtiter plates (Dynatech, South Windham, ME) overnight at 4°C. Plates were washed 4 times with PBS containing 0.5% Tween 20 (Sigma) and blocked with PBS/0.5% Tween 20 containing 5% chicken serum (Gibco, Grand Island, NY). Plates were washed once and then GST π was added for 2 hr at 4°C. Plates were washed 4 times and incubated with purified rabbit antisera to GST π (100 ng/well) for 1 hr at 4°C. After 3 washes peroxidase-labelled goat anti-rabbit immunoglobulin (Kirkegaard and Perry, Gaithersburg, MD, 1 μg/ml) was added to the plates for 1 hr at 4°C followed by 4 washes and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt (ABTS) substrate.

Immunoprecipitation studies

Hybridoma supernatants were tested for their ability to immunoprecipitate GST π in a radioimmunoassay. GST π was labelled with ^{125}I (Amersham, Arlington Heights, IL) (Marchalonis, 1969). One milliliter of hybridoma supernatant was incubated with 200,000 cpm of ^{125}I-labelled GST π overnight at 4°C. Immune complexes were isolated on protein A-sepharose CL-4B (Pharmacia) to which rabbit anti-mouse immunoglobulin was bound. Immune complexes were then quantitated in a gamma counter. In other experiments, cells were metabolically labelled with ^{35}S methionine overnight, cell detergent extracts were immunoprecipitated with MAb AGST 1, and labelled proteins resolved by SDS-PAGE (Kantor *et al.*, 1987).

Immunoaffinity purification of GST π

MAb AGST 1 was purified by adsorption to protein G Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Purified antibody was coupled to protein G Sepharose 4B by the covalent cross-linker, dimethyl pimelimidate (Pierce) according to the method of Schneider *et al.* (1982). The human colon carcinoma cell line, HT-29, was used as a source of GST π. HT-29 cells (4×10^9) stored at −70°C were lysed for 1 hr at 4°C in 200 ml of 1% Nonidet P-40 (NP-40, Fluka, Happauge, NY) in 0.05 M Tris-HCl pH 7.4, 400 mM NaCl, 10 mM EDTA, 10 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 0.02 μg/ml ovomucoid trypsin inhibitor, 1 μg/ml antipain, 1 μg/ml chymostatin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin (Sigma). The lysates were centrifuged at 18,000 *g* for 1 hr to remove nuclei and insoluble cellular material. The supernatant was pre-cleared in a batch procedure with mouse IgG bound to CnBr-activated Sepharose 4B overnight at 4°C. The mouse IgG Sepharose 4B was sedimented by centrifugation at 1,000 *g* for 5 min. The pre-cleared supernatant was then adsorbed to MAb AGST 1 protein G Sepharose in a batch procedure overnight at 4°C. For SDS-PAGE analysis, immunomatrix was washed with solubilization buffer with 0.1% SDS and eluted with sample buffer, then electrophoresis was performed on 4–20% acrylamide gradient gels (Novex, Encinitas, CA) according to Laemmli (1970).

Normal and tumor tissue specimens

Normal and tumor surgical biopsies were obtained from patients before therapy or during treatment at the Regina Elena Cancer Institute, Rome, Italy. Upon removal, tissue specimens were divided into 2 parts; one was processed for routine histological examination, the other was snap-frozen in liquid nitrogen. Cryostat sections, 4-μm thick, were heat-fixed at 37°C for 1 hr. This fixation was found to optimally preserve the serological reactivity of MAb 7C7. Sections were immediately used in immunofluorescence (IIF) or immunoperoxidase (IIP) tests, or stored at −20°C for up to 6 months. Under these storage conditions no appreciable changes in reactivity of MAb 7C7 with tissues was observed.

Immunohistochemical assays

IIF on tissue sections was performed as described by Natali *et al.* (1981) employing MAb 7C7 at concentrations of 50–100 μg/ml. Fluorescein-labelled $F(ab')_2$ fragments of a rabbit anti-mouse antiserum (Sorin, Saluggia, Italy) were extensively absorbed with packed AB-Rh^+ human red blood cells and with insolubilized normal human plasma (Avrameas and Ternynck, 1969). The absorbed antiserum was used at the concentration of 250 μg/ml. IIP staining of tissue sections was performed with a Vectastain kit (Vector, Burlingame, CA) using 3 amino-ethylcarbazole as the chromogenic substrate for 10 min at room temperature. Tissue sections were counterstained with Mayer's hematoxylin for 8–10 min (Hsu *et al.*, 1981). Specificity of MAb 7C7 reactivity in immunohistochemical assays was determined by comparison with tissues stained with a mouse IgM myeloma protein, MOPC 104 E (Sigma). Additionally, absorption of MAb 7C7 with purified GST π abolished antibody reactivity in immunohistochemical assays.

RESULTS

Production of MAb to GST π

MAb were produced to GST π isolated from the adriamycin-resistant human breast carcinoma cell line, ADr^RMCF-7. These antibodies all reacted by ELISA with GST π both from ADr^RMCF-7 cells and human placenta but not with GST α or μ isozymes (data not shown). These MAb, however, were all IgM antibodies and their binding to GST π did not affect its enzymatic activity, nor did these antibodies immunoprecipitate GST π or react with the enzyme in Western blot analysis (data not shown). We thus sought to produce MAb to other epitopes on the GST π molecule. Barclay and Smith (1986) have shown that antibodies with desired specificities could be selected by immunization of mice with antigen and antibodies of unwanted specificities. Thus, we immunized mice with GST π from placenta and 1–2 mg of MAb 7C7.

Characterization of MAb to GST π

Isotype determinations of MAb AGST 1–5 with mouse monoclonal typing reagents revealed that these antibodies were all of the IgG_1 subclass.

MAb were incubated with blots containing transferred GST π, α, and μ molecules; MAb AGST 1 reacted with a single protein band corresponding to GST π (Fig. 1, lane 4) but not

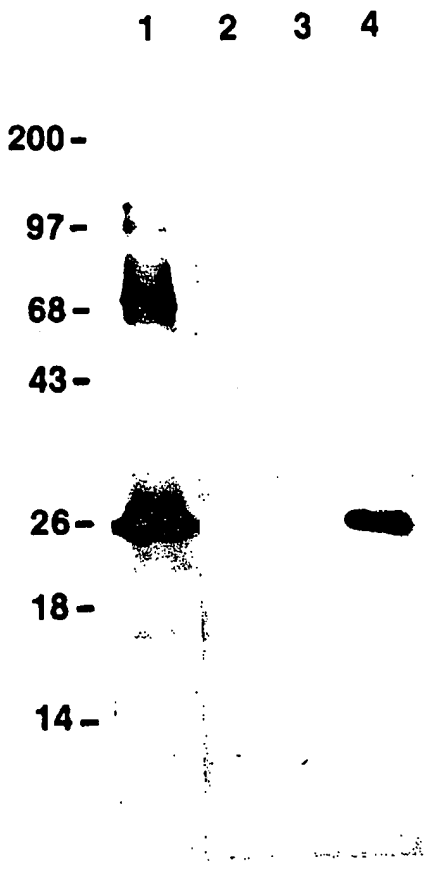


FIGURE 1 – Characterization of MAb AGST 1 specificity in Western blot analysis. Purified preparations of GST π (lanes 1, 4), μ (lane 2), and α (lane 3) were separated by SDS-PAGE under reducing conditions, transferred to Nytran, incubated with MAb AGST 1 (lanes 2–4) or rabbit antisera to GST π (lane 1) and visualized with peroxidase-conjugated second antibodies and substrate.

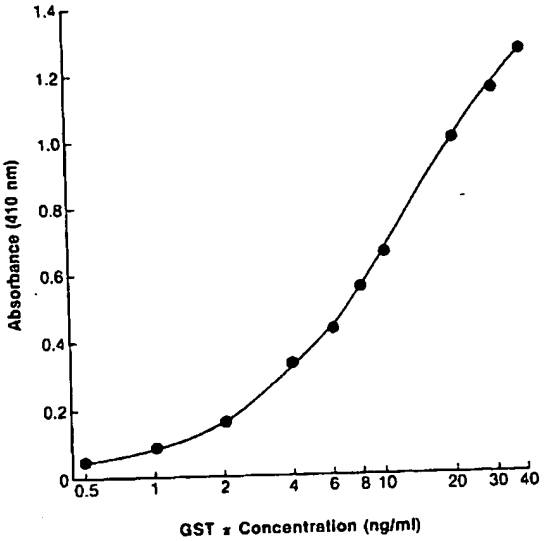


FIGURE 2 – A calibration curve for enzyme immunoassay of GST π. Sandwich ELISA using MAb AGST 1 as a capture antibody and rabbit antisera to GST π as detector antibody. Bound antibody was quantitated using peroxidase-labelled goat anti-rabbit immunoglobulins.

with GST μ or α (lanes 2, 3). Thus, MAb AGST 1 is an isozyme-specific antibody. Polyclonal rabbit antisera to GST π (lane 1) and AGST 2 (results not shown) produced similar results to MAb AGST 1.

MAb AGST 1 was further characterized by measuring its binding to purified GST π in the ELISA assay at different antibody concentrations. The binding curve obtained from this assay displayed half-maximal binding at an antibody concentration of 2.8×10^{-8} M (results not shown). The affinity of MAb AGST 1 for the antigen is thus moderate.

Development of an enzyme immunoassay to quantitate GST π

In order to quantitate GST π levels in cells, tissues, and biological fluids we developed a sandwich-type enzyme immunoassay. MAb AGST 1 was used as the "capture" antibody and rabbit antiserum to GST π was used as the "detector" antibody. This assay permits the quantitation of GST π between 0.5–40 ng/ml (Fig. 2). GST π from as few as 100 human cervical carcinoma cells, HeLa, could be detected with this assay (results not shown).

Immunoprecipitation and immunoaffinity purification of GST π

Hybridoma supernatants that reacted with GST π in ELISA were tested to assess whether they would immunoprecipitate ^{125}I-labelled GST π. Two MAbs, AGST 1 and 2, immunoprecipitated GST π (results not shown). Previous studies (Batist *et al.*, 1986) have shown that enzymatically, ADr^{R}MCF-7 cells display high levels of anionic GST and some cationic GST, while the parental drug-sensitive cells MCF-7 display only cationic enzymatic activity. MCF-7 and ADr^{R}MCF-7 cells were labelled with ^{35}S-methionine and GST π immunoprecipitated with MAb AGST 1. Figure 3*a* demonstrates the immunoprecipitation of a protein of M_r 23,000 from ADr^{R}MCF-7 cells (lane 4) with no protein immunoprecipitated from MCF-7 cells (lane 2). The immunoprecipitation of this 23-kDa protein could be prevented by pre-incubating the ADr^{R}MCF-7 detergent extract with 1 mg of purified GST π before addition of the MAb (results not shown). Large-scale preparations of GST π could be routinely prepared from 4×10^9 HT-29 cells. Cells were solubilized in 1% NP-40 and the clarified lysate was pre-absorbed with an IgG_1 affinity matrix followed by MAb AGST 1 cross-linked to protein G Sepharose. Figure 3*b* demonstrates an SDS-PAGE gel of one such immunoaffinity purification. Under non-reducing conditions (lane 3) a major protein of M_r 23,000 (GST π) and a minor protein of M_r 21,500 are seen. Western blot analysis (results not shown) with MAb AGST 1 demonstrated reactivity with both the M_r 23,000 and 21,500 proteins. Thus, although protease inhibitors have been added to buffers used in immunopurification, the M_r 21,500 protein may represent a proteolytic fragment of GST π. Immunoaffinity purification of GST π resulted in a yield of 20% based upon GST π concentrations in the starting cell lysate (90 μg GST π/120 mg protein) and following immunodepletion (72 μg GST π/120 mg protein) as determined by enzyme immunoassay.

Functional studies with AGST MAb

MAbs to GST π were tested to determine their effect on the enzymatic activity of GST π with the substrate 1-chloro-2,4 dinitrobenzene and glutathione. These experiments demonstrate that MAb AGST 1 and 2 (Fig. 4, results not shown for MAb AGST 2) displayed a dose-dependent inhibition of enzymatic activity of GST π. A 50–70% inhibition of enzymatic

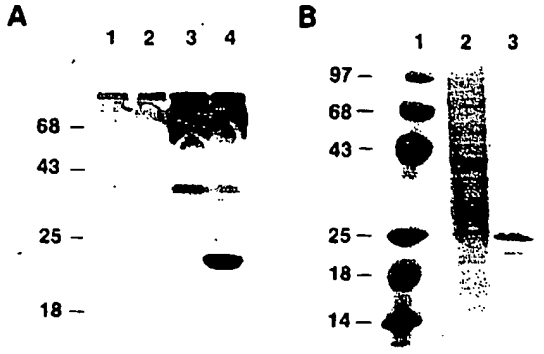


FIGURE 3 – *(a)* Autoradiographs of GST π immunoprecipitated by MAb AGST 1 from a human breast carcinoma cell line. Cell extracts of ^{35}S-methionine labelled human breast carcinoma cell line, MCF-7 (lanes 1, 2) and a doxorubin-resistant variant, ADr^{R}MCF-7 (lanes 3, 4) were immunoprecipitated with MAb AGST 1 (lanes 2, 4) or a mouse IgG$_1$ myeloma protein MOPC-21 (lane 1, 3). Labelled proteins were resolved by SDS-PAGE and the gel was processed for fluorography. *(b)* SDS-polyacrylamide gel electrophoresis of GST π isolated from cell extracts by immunoaffinity chromatography. NP-40 lysate from 4 × 10^9 HT-29 cells was absorbed with an IgG$_1$ matrix followed by a MAb AGST 1 affinity matrix. Protein(s) were eluted by boiling with non-reducing SDS-PAGE sample buffer, analyzed on a 4–20% polyacrylamide gel and visualized by staining with Coomassie brilliant blue. Lane 1; molecular weight standards, lane 2; HT-29 lysate (40 μg) and lane 3; material eluted from MAb AGST 1 matrix.

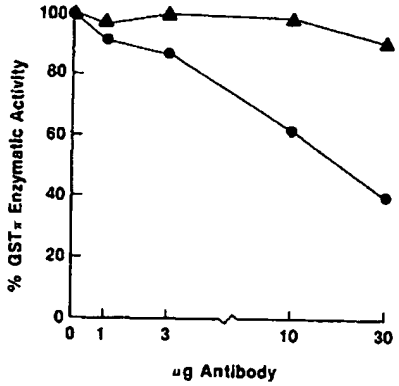


FIGURE 4 – MAb AGST-1-induced inhibition of enzymatic activity of GST π. Solutions containing 1 μg of GST π in 50 mM potassium phosphate buffer pH 6.9 were incubated with the indicated amounts of purified MAb to GST π; MAb AGST 1 (●) or MAb AGST 3 (▲) for 2 hr at 22°C. Enzymatic activity was determined with 1-chloro-2,4 dinitrobenzene as a substrate.

activity was achieved with 30 μg of antibody. No inhibition of enzymatic activity was found in studies using MAb AGST 3 (Fig. 4) or MAb AGST 4–6 (results not shown). Additionally, rabbit polyclonal antibodies to GST π and GST π peptides did not inhibit enzymatic activity (results not shown), demonstrating that MAb AGST 1 reacts with a unique epitope on the GST π molecule.

Localization of the MAb AGST 1 epitope on GST π

We have shown in previous sections of this paper that MAb AGST 1 inhibits the enzymatic activity of GST π by 50–70%. In order to map the functionally important domain of the GST π molecule defined by MAb AGST 1, we have used proteases to generate immunologically active peptides. In initial experiments, GST π was digested with a variety of proteases and the peptides were immunoprecipitated with MAb AGST 1. It became apparent that proteolysis of the GST π molecule with any of a number of proteases (*Staphylococcus aureus* V8, chymotrypsin, trypsin, endoproteinases ASP-N and LYS-N), even under very limited conditions, destroyed the MAb AGST 1 epitope (results not shown). These observations support the conclusion that MAb AGST 1 recognizes a discontinuous or conformational epitope on GST π.

As proteolysis of GST π has not provided an approach to define the MAb AGST 1 epitope, we turned to the use of antipeptide antibodies. Moscow *et al.* (1989), from structural analyses of human GST α and π, have found 2 regions of homology based upon amino acid sequence (GST π amino acid residues 53–92 and 170–179). Ketterer *et al.* (1988), from structural analysis of GST of phylogenetically unrelated organisms, have found a conserved heptadecapeptide sequence beginning about 70 residues from the N-terminus (Taylor *et al.*, 1987). This sequence contains an arginine residue which has been proposed as a point of glutathione binding. As MAb AGST 1 partially inhibits GST π enzymatic activity, it is possible that the MAb AGST 1 epitope maps to this structurally important region. To test this hypothesis, we generated rabbit antisera to a number of GST π peptides; peptide 1, amino-acid residues 53–72; peptide 2, 66–85; peptide 3, 80–99; and peptide 4, 170–191. These antipeptide antibodies react specifically with their respective peptides and with GST π in ELISA and Western blot analysis (results not shown). These antipeptide antibodies were tested for their ability to block the binding of MAb AGST 1 to GST π in ELISA. Among the antipeptide antibodies, only antisera to peptide 1 caused the blocking of MAb AGST 1 binding (Fig. 5). Our data suggest that the MAb AGST 1 binding site on GST π includes amino acid residues 53–72. Although this region of the molecule includes a putative glutathione binding region, pre-incubation of GST π-coated plates with glutathione did not inhibit binding of MAb AGST 1 (results not shown). However, this is not surprising considering the small size of GSH (M_r 307.3) and its weak affinity for GST π (dissociation constant ~150 μM) compared to that of MAb AGST 1 (2.8 × 10^{-8} M).

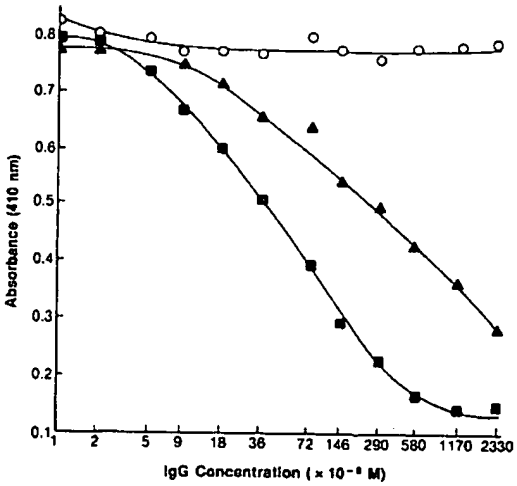


FIGURE 5 – Competition for MAb AGST 1 binding to GST π by antibodies to peptide 1 and GST π. Various concentrations of anti-P1 (▲), anti-GST π (■), and normal rabbit immunoglobulins (○) were tested in ELISA for their ability to block MAb AGST 1 binding.

Immunohistological studies

Testing of normal adult tissues with MAb 7C7 revealed no detectable staining in brain-cortex, testis, smooth muscle, cartilage, peripheral blood lymphocytes, myocardium or choroidal plexus over a wide range of MAb concentrations (Table I). All other normal tissues tested displayed varying degrees of staining. Clear, although variable, staining was observed in the epidermis in the epithelium of sweat and sebaceous glands and hair follicles, breast, thyroid, stomach, biliary ducts, bronchi and bronchial glands, transitional epithelium, prostate and thymic medulla. Epithelia from colon, rectum and endometrium were stained predominantly in their apical portions. Cytoplasmic staining was observed in proximal and distal renal tubules, endocrine and exocrine pancreas, parotid and esophagus. Furthermore, MAb 7C7 stained glomerular tufts and Bowman's capsule, red pulp splenic sinuses and vascular endothelia heterogeneously. Liver biliary ductules were strongly positive while hepatocytes were weakly positive to negative in most tissue samples. Weak staining of undefined cells was seen in peripheral nerve, perimysium and lung alveoli. The trophoblast layer of the placenta displayed prominent staining.

Testing of a total of 119 tumor samples of different histotypes revealed that GST π was widely detectable, independent of tumor type or degree of differentiation (Table II). GST π was found in tumors arising from kidney, ovary, lung, stomach, urinary bladder, colon, breast, liver, melanocytes, skin, brain, endometrium, prostate, testis, cervix and soft tissues. Although the staining of individual tumor cells in these tissue samples was heterogeneous, almost all tumor samples analyzed were found to express GST π. Additionally, there was a general increase in GST π expression in neoplastic tissues compared with normal adjacent tissues.

The potential usefulness of GST π as a neoplastic marker can be determined from analysis of the preceding immunohistological data. Thus, although GST π is widely distributed in human tissues, there are several tissues which express little to no GST π. GST π levels in normal brain and superficial cervix (Fig. 6) are undetectable by immunohistochemical procedures. GST π is, however, present in tumors arising from these tissues (Fig. 7) as well as in fibrosarcomas and chondrosarcomas. A distinctive alteration of GST π distribution in malignant transformation was observed in carcinomas of the colon, rectum and endometrium. Only the apical portions of normal colon, rectum and endometrial epithelium were stained with MAb 7C7, while in carcinomas of these tissues MAb 7C7 reacted in a broad staining pattern with the majority of neoplastic cells. Thus, GST π also appears to be a useful immunohistological marker for cancers of these tissues. The lack of information regarding the reactivity of MAb 7C7 with melanocytes, adipocytes and normal bone prevents us from stating whether GST π expression by melanoma, liposarcoma and osteosarcoma may be useful as a neoplastic marker in these tissues.

TABLE II – IMMUNOHISTOLOGICAL REACTIVITY OF MAb 7C7 WITH TUMOR TISSUES

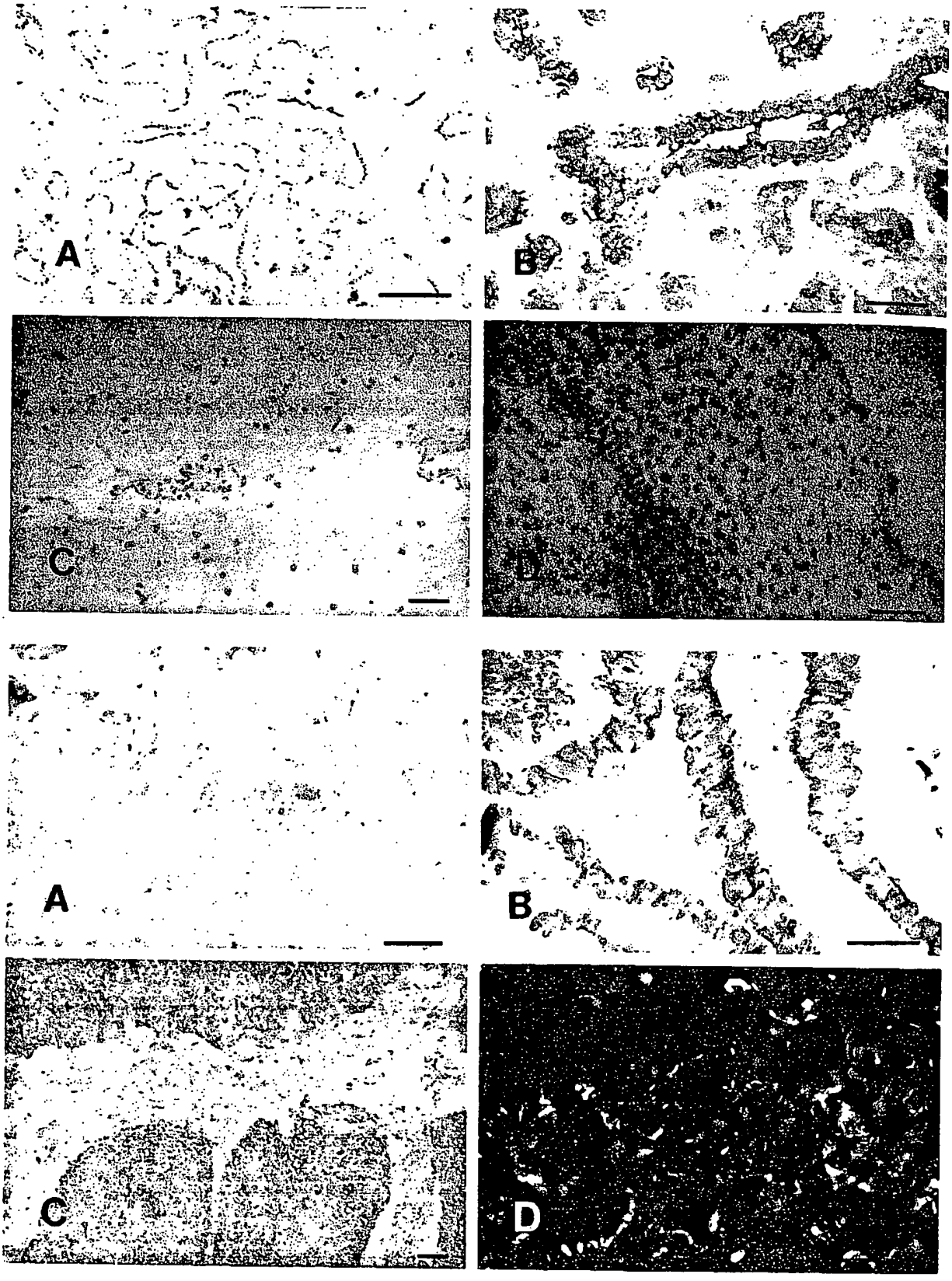
Tumor source	Number positive/number tested
Kidney	6/7
Ovary (mixed histotype)	8/8
Lung (mixed histotype)	13/14
Stomach	8/8
Urinary bladder	5/5
Colon	12/13
Breast	12/13
Liver	3/4
Melanocytes	6/6
Skin (mixed type)	4/4
Brain	9/9 glioma
	6/7 meningioma
	2/2 medulloblastoma
	1/1 ependymoma
Endometrium	2/2
Prostate	2/2
Testis	2/5 embryonal carcinoma
	embryonal teratocarcinoma
Cervix	2/2
Soft tissues	2/3 liposarcoma
	2/2 fibrosarcoma
	1 osteosarcoma
	1 chondrosarcoma
Total	109/119

DISCUSSION

Immunochemical techniques have proved extremely useful in the study of enzyme structure and function. The advantages of the immunological approach arise from the ability both to

TABLE I – IMMUNOHISTOLOGICAL REACTIVITY OF MAb 7C7 ON NORMAL ADULT TISSUES

Undetectable	Low expression	Medium expression	High expression
Brain cortex	Skeletal muscle (perimysium)	Peripheral nerves	Pancreas (exocrine)
Testis	Colon (apical portion of the epithelium)	Skin: epidermis, sweat and sebaceous glands	Parotid ductal and glandular epithelium
Smooth muscle	Cervix (basal layer)	Breast epithelium	Prostatic epithelium
Cartilage	Liver hepatocytes	Spleen (red pulp sinuses)	
Peripheral blood lymphocytes (3 cases)	Pancreas (endocrine)	Thyroid epithelium	
Myocardium		Stomach (superficial and deep glands)	
Choroidal plexus		Kidney (glomeruli, proximal and distal tubules, Bowman's capsule)	
Cervix (superficial layer)		Placental epithelium	
		Liver biliary ductules	
		Ovary (celomic epithelium)	
		Endothelium	
		Esophagus	
		Endometrium	
		Thymus (medullary portion)	
		Lymph nodes	
		Duodenum	
		Transitional epithelium	
		Lung (bronchial epithelium, alveolar walls)	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generate a large repertoire of specific MAb to an enzyme, each with its own distinctive properties, and to use amino acid sequence data to produce peptide-specific hetero-antisera. In this study, we have elicited a series of MAb and peptide-specific antisera against the GST π isozyme and characterized them. All antibodies were specific to GST π, a result which reflects the limited amino acid homology between the GST isozymes. This non-homologous primary structure is surprising because all 3 isozymes exhibit similar catalytic properties and react with an overlapping series of substrates.

This report describes the production of MAb to human GST π using an immunization procedure (Barclay and Smith, 1986), in which mice were immunized with GST π and an IgM MAb to GST π. This immunization procedure resulted in the production of MAbs that inhibited enzymatic activity and were useful in biochemical analysis. Barclay and Smith (1986) used immunization of BALB/c mice with *Dictyostelium amoebae* aggregating cells and polyclonal BALB/c antiserum raised against undifferentiated cells to obtain antibodies specific for differentiation antigens. These investigators suggested that immunization with antibodies of unwanted specificities induces the synthesis of anti-idiotype antibodies, which prevents the synthesis of antibodies with identical or cross-reacting idiotypes. Our own work suggests that this approach might be useful in order to obtain MAbs to different epitopes on a single molecule.

MAbs AGST 1 and 2 inhibit the enzymatic activity of GST π towards the substrate 1-chloro-2,4 dinitrobenzene. MAbs with high specificity and affinity are required to inhibit enzymatic activity. The lack of inhibitory activity of the other 4 MAbs (AGST 3–6) correlates with their lack of activity in the immunoprecipitation assay. The isozymes of GST probably facilitate bimolecular reactions, at least in part, by serving as a scaffold to bring GSH and substrate into close physical proximity so that a spontaneous nucleophilic attack of the sulfhydryl group of GSH on the electrophilic center of the substrate can occur (Mannervik, 1985). The binding of MAb AGST 1, 2 may inhibit the enzymatic activity of GST π by altering its overall structure or conformation or by inhibiting GSH or substrate binding. A heptadecapeptide sequence beginning about 70 residues from the N-terminus of GST has been suggested to be a conserved GSH binding site on GSTs (Taylor *et al.*, 1987). We have now partially defined the region of MAb AGST 1 and 2 binding on GST π to include this putative substrate binding sequence. These results do not preclude the existence of other molecular sites important in GSH binding or to enzymatic activity. Tamai *et al.* (1990) have shown that the 47th residue (cysteine) from the N-terminus of GST-P was preferentially modified by oxidation and resulted in the loss of GSH binding capacity of GST-P.

MAb which can neutralize the enzymatic activity of GST π might be useful in defining the physiological role of this enzyme. MAb AGST 1 and 2 could be used in micro-injection studies. Although these antibodies can only partially inhibit enzymatic activity using purified enzyme with the substrate 1-chloro-2,4 dinitrobenzene, this inhibition is not sufficient to predict the effect of these antibodies on cellular physiology.

The observations that the π class of GST may provide a useful marker for neoplastic transformation in certain cancers and for resistance of neoplastic cells to certain anti-cancer agents has prompted these investigations into the expression of this enzyme in a broad panel of normal human tissues and the tumors arising from these tissues. Immunohistological studies with MAb 7C7, using heat-fixed cryostat tissue sections, have shown a wide distribution of GST π. Many normal tissues examined expressed GST π, with the exceptions of brain-cortex, testis, smooth muscle, cartilage, peripheral blood lymphocytes, myocardium and choroidal plexus. Previous studies using enzymatic analysis have reported anionic or acid GST in adrenal, ovary, kidney, lung, erythrocytes, platelets, thyroid, placenta, lung, heart, spleen, kidney, pancreas and liver (Ketterer *et al.*, 1988; Sherman *et al.*, 1983; Lascalzo and Freedman, 1986; Del Boccio *et al.*, 1987; Tateoka *et al.*, 1987). Our finding of the lack of expression of GST π in heart and testis is in contrast to other studies (Ketterer *et al.*, 1988; Sherman *et al.*, 1983; Tateoka *et al.*, 1987) in which GST isozymes resolved by isoelectric focusing and chromatographic procedures were enzymatically assayed. The widespread tissue distribution of GST π suggests that this enzyme is essential to normal cellular physiology. However, definition of the major biological role of GST π as well as its endogenous substrates remains to be resolved.

The immunohistochemical results of the present study show a cytoplasmic staining pattern of most human tissues with MAb 7C7. However, in a limited number of tissues, a membrane staining pattern was also seen. It is possible that the mild fixation procedures used in these studies allowed for the membrane visualization. Subsequent investigations performed with MAb AGST 1 and polyclonal antisera on formalin-fixed cervical biopsy samples demonstrated GST π in the nucleus and cytoplasm of some samples (data not shown). Subcellular fractionation studies performed with the human colon carcinoma cell line, HT-29, revealed GST π immunoreactivity in several subcellular fractions, including those containing the cytoplasm, membrane and nucleus (results not shown). Thus, GST π appears to be present in several subcellular locations. It is tempting to speculate that the enzyme may subserve different functions in different sites. Bennett *et al.* (1986) have reported that GST Yb, a μ-class isozyme, is a DNA-binding nonhistone protein found in transcriptionally active nuclear regions and may play an important role in either biotransforming electrophilic compounds or regulating gene expression. Other studies have implied that GSTs found in the nucleus may represent a new class of DNA repair enzymes that work in concert with other DNA repair molecules (Ketterer *et al.*, 1987).

Our report confirms that GST π is present in virtually all primary human tumors including tumors of the kidney, ovary, lung, stomach, urinary bladder, colon, breast, liver, melanocytes, skin, brain, endometrium, prostate, testis, cervix and soft tissues. GST π expression in tumor tissues is heterogeneous at the cellular level. We have also found that GST π levels are increased in neoplastic tissues compared to their normal tissue counterparts. Other investigators have also reported expression of GST π in human tumors. GST π has been demonstrated immunochemically in hepatocellular carcinoma (Sherman *et al.*, 1983; Soma *et al.*, 1986), metastatic melanoma (Mannervik *et al.*, 1987), prostate carcinoma (Tew *et al.*, 1987) and colon and esophageal cancers (Tsuchida *et al.*, 1989); immunohistologically in cancers of the colon, stomach, pancreas and uterine cervix (Kodate *et al.*, 1986; Shiratori *et al.*, 1987; Tsutsumi *et al.*, 1987; Sato *et al.*, 1987) and by isoelectric focusing in renal cancer (Di Ilio *et al.*, 1987) and a panel of 17 tumor samples (Shea *et al.*, 1988). Moscow *et al.* (1989) have shown enhanced GST π mRNA expression in 12 out of 12 colon cancer samples compared to normal tissues

FIGURE 6 – Distribution of GST π detected with MAb 7C7 by indirect avidin-biotin IIP staining on heat-fixed cryostat sections of normal tissues. MAb 7C7 homogeneously stains the walls of splenic sinuses with a punctate pattern *(a)* and heterogeneously stains the plasma membranes of mammary epithelium *(b)*. No stain is detectable on brain cortex *(c)* or uterine cervix *(d)*. Bar, 50 μm.

FIGURE 7 – Indirect avidin-biotin IIP and IIF staining patterns of MAb 7C7 on tumors. MAb 7C7 heterogeneously stains tumor cells of a renal adenocarcinoma *(a)*, and reacts homogeneously with cells of an ovarian carcinoma *(b)* and a carcinoma of the uterine cervix *(c)*. A dotted heterogeneous stain is detectable by IIF in glioma cells *(d)*. Bar, 50 μm.

obtained from surgical margins. These findings of enhanced GST π expression in cancers suggest that the GST π gene is activated during malignant transformation. This transformation may be mediated by any of a number of agents, including chemical carcinogens (Sato *et al.*, 1984; Satoh *et al.*, 1985; Tatematsu *et al.*, 1985; Moore *et al.*, 1985; Obara *et al.*, 1986), *ras* oncogene (Li *et al.*, 1988), and possibly viruses (Shiratori *et al.*, 1987; Durst *et al.*, 1983; Boshart *et al.*, 1984). Thus, the induction or enhanced expression of GST π reflects the activation of common pathways associated with transformation. The contribution of GST π to the transformed phenotype is at present unknown.

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